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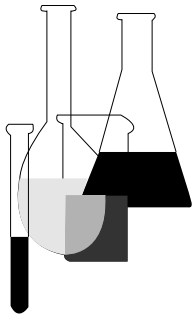
Health Effects Test Guidelines OPPTS 870.1350 Acute Inhalation Toxicity With Histopathology



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 870.1350 Acute inhalation toxicity with histopathology.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** This is a new guideline developed in the Office of Research and Development.

(b) **Purpose**. In the assessment and evaluation of the potential human health effects of chemical substances, it is appropriate to test for acute inhalation toxic effects. The goals of this test are to characterize the exposure-response relationship for sensitive endpoints following acute exposure and to characterize toxicologic response following acute high exposures. The latter is of particular concern in relation to spills and other accidental releases. This testing is designed to determine the gross pathology resulting from acute inhalation exposure to a substance. In addition, because toxic effects on the respiratory tract are of particular concern following inhalation exposure, several indicators of respiratory toxicity consisting of histopathology on fixed tissue and evaluation of cellular and biochemical parameters in bronchoalveolar lavage fluid should be employed. This acute testing should be augmented by the respiratory sensory irritation assay in mice (ASTM, 1984). The respiratory histopathology consists of specialized techniques to preserve tissues of the respiratory tract in order to allow detailed microscopic examination to identify adverse effects of chemical substances on this organ system. The bronchoalveolar lavage is designed to be a rapid screening test to provide an early indicator of pulmonary toxicity by examining biochemical and cytologic endpoints of material from the lungs of animals exposed to potentially toxic chemical substances. The mouse respiratory sensory irritation assay is a non-invasive assay designed to detect sensory irritation during exposure to a chemical substance (ASTM, 1984). These acute tests are designed to assess the relationship, if any, between the animals exposed to the test substance, the incidence and severity of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects. These acute tests are not intended to provide a complete evaluation of the toxicologic effects of a substance, and additional functional and morphological evaluations may be necessary to assess completely the potential effects produced by a chemical substance. Additional tests may include longer-term exposures, or more in-depth evaluation of specific organ systems as indicated by signs of toxicity following acute exposure.

(c) **Definitions.** The following definitions apply to this test guideline.

Aerodynamic diameter refers to the size of particles or aerosols. It is the diameter of a sphere of unit density that behaves aerodynamically (has the same settling velocity in air) as the particle of the test substance. It is used to compare particles of different size, shape and density, and

to predict where in the respiratory tract such particles may be primarily deposited.

Exposure response is the relationship between the exposure concentration and the measured response expressed as a group mean in the case of a continuous variable or as incidence in the case of a quantil variable.

Geometric standard deviation (GSD) is a dimensionless number equal to the ratio between the mass median aerodynamic diameter (MMAD) and either 84% or 16% of the diameter size distribution (e.g., MMAD = 2 μm; 84% = 4 μm; GSD = 4/2 = 2.0.) The MMAD, together with the GSD, describe the particle size distribution of an aerosol.

Lower respiratory tract consists of those structures of the respiratory tract below the larynx.

The *mass geometric mean aerodynamic diameter or the mass median aerodynamic diameter* (MMAD) is the calculated aerodynamic diameter that divides the particles of an aerosol (a gaseous suspension of fine liquid or solid particles) in half, based on the weight of the particles. By weight, 50% of the particles will be larger than the MMAD and 50% of the particles will be smaller than the MMAD.

Particle regional deposition is the region of the respiratory tract where particles of different sizes deposit. Particles between 5 and 30 μm mainly deposit in the nasopharyngeal region by inertial impaction, particles between 1 and 5 μm deposit by sedimentation in the tracheal/bronchial/bronchiolar/alveolar regions, and particles of 1 μm or less deposit in the alveolar region by diffusion.

Respiratory effects are any adverse effects on the structure or functions of the respiratory system related to exposure to a chemical substance.

Sensory airway irritation is any sign that a chemical substance is stimulating the nerves of the respiratory tract as identified by the characteristic pause during expiration in the respiratory sensory irritation test.

Toxic effects are any adverse changes (a change that is statistically or biologically significant) in the structure or function of an experimental animal as a result of exposure to a chemical substance.

Upper respiratory tract consists of those structures of the respiratory tract above the larynx.

(d) **Principle of the test method**. The test substance is administered to several groups of experimental animals; one concentration level and duration being used per group. Bronchoalveolar lavage shall be used to evaluate early effects on the respiratory system by examining changes in the content of the lavage fluid of the lung. At 24 hours following exposure,

the animals shall be sacrificed and necropsied, and tissue samples from the respiratory tract and other major organs will be prepared for microscopic examination. The exposure levels at which significant toxic effects on the respiratory organ system are produced are compared to those levels that produce other toxic effects. As triggered by the results of the 4-hour test, additional exposure periods of 1 hour and 8 hours will be required to determine the effect of exposure time on the toxicity observed.

(e) **Test procedures**—(1) **Animal selection**—(i) **Species**. In general, the laboratory rat and mouse should be used. Under some circumstances, other species, such as the hamster or guinea pig, may be more appropriate, and if these or other species are used, justification should be provided.

(ii) **Strain**. If rats and mice are used, the use of the F344 rat and the B6C3F1 mouse is preferred to facilitate comparison with existing data.

(iii) **Age.** Young adults shall be used. The weight variation of animals used in a test should not exceed ± 20 % of the mean weight for each species.

(iv) **Sex.** Equal numbers of animals of each sex shall be used for each dose level. The females shall be nulliparous and nonpregnant.

(v) **Health status.** Body weight and feed consumption are not sufficient indicators of the health status of animals prior to initiating an inhalation toxicity study. Prior to initiating the study, animals shall be monitored for known pathogenic rodent viruses as determined by conventional microbiological assays (e.g., serology). In addition, a few animals shall be sacrificed prior to exposure, lung lavage fluid shall be evaluated for polymorphonuclear leukocytes (PMNs), and biochemical parameters shall be evaluated as in the lung lavage test described in paragraph (e)(12) of this section to assure the health of the animals. PMNs should be less than 1% of lavage cells. The animals shall be free from pathogens at the start of exposure.

(2) **Number of animals.** At least five males and five females shall be used in each concentration/duration and control group. Animals shall be randomly assigned to treatment and control groups.

(3) **Control groups.** A concurrent control group is required for all tests. The control group shall be a sham treated group. Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test group animals. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group shall be used.

(4) **Concentration level and concentration selection.** For the 4-hour study, at least three concentrations shall be used in addition to the control group. Ideally, the data generated from the test should be sufficient to

produce an exposure-effect curve. The concentrations can either be linearly or logarithmically spaced depending on the anticipated steepness of the concentration-response curve. A rationale for concentration selection should be provided to indicate that the selected concentrations will maximally support detection of concentration-effect relationship. The high concentration should be clearly toxic or a limit dose, but should not result in an incidence of fatalities that would preclude a meaningful evaluation of the data. The lowest concentration should define a no-observed-adverse-effects level (NOAEL).

(i) **Limit dose.** The high concentrations need not be greater than 5 mg/L, or for aerosol and particulate concentrations, concentrations that cannot maintain a size distribution less than or equal to 4 μm (MAD) (i.e., a particle size distribution that permits deposition throughout the respiratory tract). If a test at an exposure of 5 mg/L (actual concentration of respirable substance) for 4 hours or, where this is not feasible, the maximum attainable concentration, using the procedures described for this study, produces no observable toxic effects, then a full study using three dose levels will not be necessary.

(ii) **1-Hour study.** If triggered, three concentrations shall be tested. These concentrations should allow for the determination of an effect level and a no-observable-adverse-effect-level.

(iii) **8-Hour study.** If triggered, three concentrations shall be tested. These concentrations should allow for the determination of an effect level and a no-observable-adverse-effect-level.

(5) **Inhalation exposure.** Animals can be exposed to the substance by either a nose only procedure or in a whole body exposure chamber.

(i) **Inhalation chambers.** The animals shall be tested in inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of at least 19% and an evenly distributed exposure atmosphere. Where a whole body chamber is used, its design shall minimize crowding by providing individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total ‘‘volume’’ of the test animals should not exceed 5% of the volume of the test chamber.

(ii) **Environmental conditions.** The temperature at which the test is performed shall be maintained at 22 °C (±2 °C). Ideally, the relative humidity should be maintained between 40% and 60%, but in certain instances (e.g., tests using water as a vehicle), this may not be practical.

(iii) **Exposure periodicity.** For acute testing, the animals shall be exposed to the test substance for at least 4 hours after the chamber has reached equilibrium. If triggered by the results of the 4 hour exposure, additional testing shall be conducted using 1 and 8 hour exposure periods.

(6) **Physical measurements.** Measurements or monitoring shall be made of the following:

(i) The rate of air flow shall be monitored continuously, but shall be recorded at least every 30 minutes.

(ii) The actual concentrations of the test substance shall be measured in the breathing zone. During the exposure period, the actual concentrations of the test substance shall be held as constant as practicable, monitored continuously, and recorded at least at the beginning, at an intermediate time, and at the end of the exposure period.

(iii) During the development of the generating system, where appropriate, particle size analysis shall be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution.

(iv) Chemical purity, where appropriate, shall be verified after the compound is aerosolized, preferably by sampling in the animals' breathing zone prior to initiating the study. This is to ensure that purity has not changed from the stock material to the breathing zone, and that degradation products have not formed.

(v) If the compound is present in a mixture, the mass and composition of the entire mixture, as well as the principal compound, shall be measured.

(vi) Temperature and humidity shall be monitored continuously, but shall be recorded at least every 30 minutes.

(7) **Food and water during exposure period.** Food shall be withheld during exposure. Water may also be withheld in certain cases.

(8) **Observation period.** The bronchoalveolar lavage and respiratory pathology shall be conducted 24 hours following exposure to allow expression of signs of toxicity. There is concern that some latency time will be required to allow migration of cells and macromolecules into the lungs following exposure, and that some pathology may require macromolecular synthesis or degradation before cell damage develops.

(9) **Gross pathology**. (i) All animals shall be subjected to a full necropsy which includes examination of orifices and the cranial, thoracic, and abdominal cavities and their contents.

(ii) At least the lungs, liver, kidneys, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future

histopathological examination: All gross lesions; brain-including sections of medulla/pons; cerebellar cortex and cerebral cortex; pituitary; thyroid/ parathyroid; thymus; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; accessory genital organs (epididymis, prostrate, and, if present, seminal vesicles); aorta; skin; gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; thigh musculature; peripheral nerve; spinal cord at three levels cervical, midthoracic, and lumbar; and eyes. Tissue preservation for the respiratory tract is described in paragraph (e)(11) of this section.

(10) **Histopathology.** The following histopathology shall be performed:

(i) Full histopathology shall be performed on the respiratory tract, liver and kidney of all animals in the control and high dose groups. The histopathology of the respiratory tract is described in paragraph (e)(11) of this section.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals, as indicated by the observations in the high dose group in this study.

(iv) Archived organs (identified as targets of toxicity from results of the 90-day study, if a 90-day study is required for this substance) in high dose animals of the 4-hour acute study should be evaluated to determine if they are also targets of acute toxicity.

(11) **Respiratory tract histopathology.** (i) Representative sections of the respiratory tract shall be examined histologically. These shall include the trachea, major conducting airways, alveolar region, terminal and respiratory bronchioles, alveolar ducts and sacs, and interstitial tissues.

(ii) Care shall be taken that the method used to kill the animal does not result in damage to the tissues of the upper or lower respiratory tract.

(iii) Four sections of the nasopharyngeal tissue shall be examined for histopathologic lesions. This shall include sections through the nasal cavity, and examination of the squamous, transitional, respiratory, and olfactory epithelia. The types and locations of lesions shall be reported using diagrams of the nose.

(iv) The laryngeal mucosa shall be examined for histopathologic changes. Sections of the larynx to be examined include the epithelium covering the base of the epiglottis, the ventral pouch, and the medial surfaces of the vocal processes of the arytenoid cartilages.

(12) **Bronchoalveolar lavage.** (i) Animals can be exposed to the substance by either a nose only procedure or in a whole body exposure chamber.

(ii) Care should be taken that the method used to kill the animal results in minimum changes in the fluid of the lungs of the test animals.

(iii) At the appropriate time, the test animals shall be killed and the heart-lung including trachea removed *in bloc*. Alternatively, lungs can be lavaged *in situ*. If the study will not be compromised, one lobe of the lungs may be used for lung lavage while the other is fixed for histologic evaluation. The lungs should be lavaged using physiological saline. The lavages shall consist of two washes, each of which consists of approximately 80% (e.g., 5 ml in rats and 1 ml in mice) of the total lung volume. Additional washes merely tend to reduce the concentrations of the material collected. The lung lavage fluid shall be stored on ice at 5 °C until assayed.

(iv) The following parameters shall be determined in the lavage fluid as indicators of cellular damage in the lungs: Total protein, cell count, and percent leukocytes. In addition, a phagocytosis assay using the procedure of Burleson (Burleson et al., 1987; Gilmour and Selgrade, 1993), paragraphs (h)(1) and (h)(2) of this section, shall be performed to determine macrophage activity.

(13) **Combined protocol.** The tests described may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(f) **Triggered testing.** If no adverse effects are seen in the 4-hour study as compared with controls, no further testing is necessary. If the 4-hour study shows positive effects in histopathology of the bronchoalveolar lavage, a 1-hour study and an 8-hour study shall be conducted. Only those systems showing positive results in the 4-hour study must be pursued in the follow-up 1-hour and 8-hour studies.

(g) **Data reporting and evaluation.** The final test report must include the following information:

(1) **Description of equipment and test methods.** A description of the general design of the experiment and any equipment used shall be provided. This shall include a short justification explaining any deviations from protocol.

(i) Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particulates, aerosols, gasses, and vapors, method of conditioning air, treatment of exhaust air, and the method of housing animals in a test chamber.

(ii) Description of the equipment for measuring temperature, humidity, and particulate aerosol concentration and size.

(iii) Exposure data shall be tabulated and presented with mean values and measure of variability (e.g., standard deviation) and should include:

(A) Airflow rates through the inhalation equipment.

(B) Temperature and humidity of air.

(C) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by the volume of air).

(D) Actual concentration in test breathing zone.

(E) Particle size distribution (e.g., MMAD with GSD), where appropriate.

(2) **Results**—(i) **General group animal data.** The following information must be arranged by test group exposure level.

(A) Number of animals exposed.

(B) Number of animals dying.

(C) Number of animals showing overt signs of toxicity.

(D) Pre- and post-exposure body weight change in animals, and weight change during the observation period.

(ii) **Counts and incidence of gross alterations observed at necropsy in the test and control groups.** Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any gross lesions were found.

(B) The number of animals affected by each different type of lesion, and the locations and frequency of each type of lesion.

(iii) **Counts and incidence of general histologic alterations in the test group.** Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any histopathologic lesions were found.

(B) The number of animals affected by each different type of lesion, and the locations, frequency, and average grade of each type of lesion.

(iv) **Counts and incidence of respiratory histopathologic alterations by the test group.** The nasal lesions report shall be accompanied by diagrams of the locations of the lesions. Data shall be tabulated to show:

(A) The number of animals used in each group and the number of animals in which any lesion was found.

(B) The number of animals affected by each different type of lesion, and the locations, frequency, and average grade of each type of lesion.

(v) **Results of the bronchoalveolar lavage study.** Data shall be tabulated to show:

(A) The amount of administered lavage fluid and recovered lavage fluid for each test animal.

(B) The magnitude of change of biochemical and cytologic indices in lavage fluids at each test concentration for each animal.

(C) Results shall be quantified as amount of constituent/mL of lavage fluid. This assumes that the amount of lavage fluid recovered is a representative sample of the total lavage fluid.

(3) **Evaluation of data.** The findings from this acute study should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlated functional findings. The evaluation shall include the relationship between the concentrations of the test substance and the presence or absence, incidence, and severity of any effects. The evaluation should include appropriate statistical analyses, for example, parametric tests for continuous data and non-parametric tests for the remainder. Choice of analyses should consider tests appropriate to the experimental design, including repeated measures. The report must include concentration-effect curves for the bronchoalveolar lavage and tables reporting observations at each concentration level for necropsy findings and gross, general, and respiratory system histopathology.

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Burleson, G.R., Fuller, L.B., Me'nache, M.G., and Graham, J.A. ''Poly (I): poly (C)-enhanced alveolar peritoneal macrophage phagocytosis: Quantification by a new method utilizing fluorescent beads.'' *Proceedings of the Society of Experimental Biology and Medicine.* 184:468–476 (1987).

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(3) Henderson, R.F., Benson, J.M., Hahn, F.F., Hobbs, C.H., Jones, R.K., Mauderly, J.L., McClellan, R.O., and Pickrell, J.A. ''New approaches for the evaluation of pulmonary toxicity: Bronchoalveolar lavage fluid analysis.'' *Fundamental and Applied Toxicology.* 5:451–458 (1985).

(4) Henderson, R.F. ''Use of bronchoalveolar lavage to detect lung damage.'' *Environmental Health Perspectives.* 56:115–129 (1984).

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(6) Renne, R.A., Gideon, K.M., Miller, R.A., Mellick, P.W., and Grumbein, S.L. ‘‘Histologic methods and interspecies variations in the laryngeal histology of F344/N rats and B6C3F1 mice.’’ *Toxicology and Pathology.* 20:44–51 (1992).

(7) Young, J.T. ‘‘Histopathologic examination of the rat nasal cavity.’’ *Fundamental and Applied Toxicology.* 1:309–312 (1981).